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Receptor Editing Can Lead to Allelic Inclusion and Development of B Cells That Retain Antibodies Reacting with High Avidity Autoantigens

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Receptor editing is a major B cell tolerance mechanism that operates by secondary Ig gene rearrangements to change the specificity of autoreactive developing B cells. In the 3-83IgH mouse model, receptor editing operates in every autoreactive anti-H-2Kb B cell, providing a novel receptor without additional cell loss. Despite the efficiency of receptor editing in generating nonautoreactive Ag receptors, we show in this study that this process does not inactivate the autoantibody-encoding gene(s) in every autoreactive B cell. In fact, receptor editing can generate allelically and isotypically included B cells that simultaneously express the original autoreactive and a novel nonautoreactive Ag receptors. Such dual Ab-expressing B cells differentiate into transitional and mature B cells retaining the expression of the autoantibody despite the high avidity interaction between the autoantibody and the self-Ag in this system. Moreover, we find that these high avidity autoreactive B cells retain the autoreactive Ag receptor within the cell as a consequence of autoantigen engagement and through a Src family kinase-dependent process. Finally, anti-H-2Kb IgM autoantibodies are found in the sera of older 3-83IgH mice, indicating that dual Ab-expressing autoreactive B cells are potentially functional and capable of differentiating into IgM autoantibody-secreting plasma cells under certain circumstances. These results demonstrate that autoreactive B cells reacting with ubiquitous membrane bound autoantigens can bypass mechanisms of central tolerance by coexpressing nonautoreactive Abs. These dual Ab-expressing autoreactive B cells conceal their autoantibodies within the cell manifesting a superficially tolerant phenotype that can be partially overcome to secrete IgM autoantibodies. The Journal of Immunology, 2005, 175: 5067–5076.

One of the fundamental tenets of immunology is that each lymphocyte expresses an Ag receptor of unique specificity. Thus, for B lymphocytes, a given B cell is activated only by a specific Ag. This allows autoreactive (self-Ag-specific) B cells to be negatively selected during maturation, and pathogen-specific B cells to be activated for fighting infections. Considerable evidence supports this postulate, because genetic and protein analyses indicate that each B cell, generally, rearranges and expresses either the maternal or the paternal derived Ig alleles, a phenomenon termed allelic exclusion (reviewed in Refs. 1–5). However, allelic exclusion has its exceptions, and rare B cells expressing either two Ig H or L chains (allelically/isotypically included B cells) have been observed in healthy human subjects and in wild-type mice (6–13). These B cells express two or more Abs (as a result of random pairing of multiple H and L chains) and, consequently, can react with multiple Ags. How and why allelically/isotypically included B cells are generated and what their Ag specificity remains unknown.

Studies spanning over a decade have established that developing B cells expressing Abs reacting with high avidity autoantigens undergo receptor editing, in which they continue to rearrange their Ig genes. These additional Ig gene rearrangements result, generally, in the inactivation of the gene(s) encoding the autoreactive Ig chains, rearrangement of a novel Ig gene, and expression of a nonautoreactive Ag receptor (BCR) that permits further B cell maturation (4, 14–16). Because of the diploid nature of the mammalian genome, receptor editing can generate B cells that maintain the genes encoding the autoantibody on one allele while rearranging the other IgH or, most likely, Igk and Igλ alleles (encoding κ- and λ-chains, respectively), possibly providing a mechanism for the generation of allelically/isotypically included B cells, including those that are autoreactive.

Work on anti-DNA IgH knock-in mice supports a role for receptor editing in the generations of allelically/isotypically included autoreactive B cells. Developing B cells in these mice undergo several cycles of receptor editing in an attempt to eliminate autoreactivity, but ultimately differentiate into mature B cells that express both an autoreactive and a nonautoreactive L chain on the cell surface (17, 18). The retention of the autoreactive Ag receptors on the cell surface suggests that these B cells manifest low avidity for their specific autoantigens, in that either the affinity of these autoantibodies for the autoantigen or the concentration of the available autoantigen is sufficiently low so as not to induce receptor internalization. Presently, it is unclear whether Ig allelic/isotypic inclusion can mediate the development of autoreactive B cells that manifest high avidity for autoantigens, such as those reactive with ubiquitous membrane-bound molecules. As a matter of fact, the development of B cells specific for ubiquitous membrane-bound autoantigens has never been observed, indicating that such high avidity autoreactive B cells always succumb to central tolerance (19–22).
To investigate whether receptor editing can induce the development of dual Ab-expressing B cells that retain the expression of a high avidity autoantibody, and whether coexpression of a nonautoreactive BCR in developing autoreactive B cells is sufficient to mediate the survival and differentiation of high avidity autoreactive B cells, we investigated the development and selection of anti-H-2K^b autoreactive B cells in the 3-83Igi mouse model. We have previously shown that in the 3-83IgI (Igh^B1-8,3-83Igi, Igk^B1-8,3-83Igi) mouse strain, in which B cells express the 3-83 Ab specific for the membrane-bound autoantigen H-2K^b, developing B cells undergo receptor editing upon autoantigen binding and generate a mature and tolerant B cell population apparently devoid of autoantibody (23–25).

In this study we demonstrate that during receptor editing, up to 20% of anti-H-2K^b B cells acquire a novel, presumably nonautoreactive, Ag receptor and develop further into splenic B cells that retain the autoreactive 3-83 Ab despite a high avidity interaction with the ubiquitous H-2K^b self-Ag. Moreover, coexpression of a nonautoreactive BCR is sufficient to mediate development of autoreactive B cells, and receptor editing is absolutely required for this event. Importantly, dual Ab-expressing autoreactive B cells appear superficially tolerant, because they retain the autoreactive Ab inside the cell, but have the potential to differentiate into 3-83 IgM-secreting plasma cells in older mice.

Materials and Methods

Mice

The 3-83IgI homozygous and 3-83IgI/+ hemizygous 3-83IgI (Igh^B1-8,3-83Igi, Igk^B1-8,3-83Igi, Igk^B1-8,3-83Igi), the B1-8His3-83Igi (Igh^H1-8,3-83Igi), and 3-83Hi (Igh^B1-8,3-83Igi, Igk^B1-8,3-83Igi) mice have been described previously (23, 26). The 3-83IgI homozygous and 3-83IgI/+ mice were genotyped on BALB/c (H-2b) genetic backgrounds, whereas the B1-8His3-83Igi mice are on a mixed C57BL/6 x BALB/c background. B1-8His3-83Igi and 3-83IgI mice were intercrossed to generate dual Ab-expressing B1-83-83Igi (Igh^B1-8,3-83Igi, Igk^B1-8,3-83Igi) mice on both H-2^d and H-2^b backgrounds. The 3-83IgI, H-2^d mouse was bred to Rag1^−/− (BALB/c; The Jackson Laboratory) mice to generate 3-83IgI, H-2^b, Rag1^−/− mice. Wild-type mice were from either BALB/c or C57Bl/6 strains. All mice were housed and bred in specific pathogen-free conditions at the Biologic Resource Center of the National Jewish Medical and Research Center. Experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Flow cytometry and Abs

Bone marrow and spleen cells were prepared and stained as previously described (27). Abs used for cell staining were against B220 (RA3-6B2; BD Pharmingen) (28), IgM (R3-3-24) (29), IgM^* (BD Pharmingen), CD3 (14.18; BD Pharmingen), CD21 (7G6; BD Pharmingen), CD22 (Lyb2; BD Pharmingen), CD23 (B3B4; BD Pharmingen), IgK (goat polyclonal; Southern Biotechnology Associates), 3-83IgI (54.1) (30), B1-8Hi (Ac146) (31), and 3-83Ex (S27) (12). Binding of cells to H-2^d and 54.1 was performed by incubating spleen cells from 3-83IgI, H-2^d mice with 40 g/ml soluble H-2K^b-IgG1 (Sigma) for 15 min in ice. Cells were then washed and stained with anti-B220 as well as anti-IgK and 54.1 Abs to detect by flow cytometry by membrane-bound IgG1 (IgK) and 54.1 molecules, respectively. The Abs were used as FITC, 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester, PE, allophycocyanin, Cy5, PerCP, and biotin conjugates. Biotinylated Abs were revealed with streptavidin-allophycocyanin (BD Pharmingen). Propidium iodide (1.25 μg/ml) was added before flow cytometric analysis. Stained cells were analyzed on a FACSCalibur (BD Biosciences) and with FlowJo 4.3 software. All cell analyses were conducted on a live lymphoid gate (based on propidium iodide staining and forward and side scatter).

Bone marrow chimeras, cell culture, and cell adoptive transfer

Bone marrow chimeric mice were generated as previously described (25) and were analyzed 5–8 wk after adoptive transfer. Spleen B220^+ B cells from bone marrow chimeras and intact mice were purified (90–96% purity) by MACS (Miltenyi Biotec) with magnetic beads conjugated to anti-B220 Abs (Miltenyi Biotec). Purified cells were either cultivated in complete DMEM/10% FBS medium supplemented with 10 μg/ml LPS (Sigma-Aldrich) or injected i.v. into untreated recipient mice (~2 × 10^7 cells/mouse). Cell-adopted transferred mice and cultured cells were analyzed by flow cytometry 1 and 2 days later, respectively, as described in the figures. Supernatants from cultured cells were analyzed by ELISA after 7 days of culture. Cell cultures with PP2 and bafilomycin were set up as follows:

Total spleen cells were cultured in complete DMEM/10% FBS medium at a concentration of 6 × 10^6/ml in 12-well plates. PP2 (Calbiochem) was added to the culture to a final 2.5 μM concentration. Control cultures were added with a similar amount of DMSO (0.1%). Cells were incubated in a 37°C incubator in 5% CO_2. Twelve hours after the initiation of culture, bafilomycin (Calbiochem) was added at a final concentration of 100 nM for another 12 h of culture. After 24 h from the beginning of culture, cells were stained with anti-B220 and anti-3-83IgI (54.1) Abs and analyzed on a FACSCalibur (BD Biosciences).

ELISA

Secreted 3-83IgM, total IgM, and IgG levels in serum and/or cell supernatants were measured by ELISA. Briefly, 96-well Nunc-Immuno plates (Nalge Nunc International) were coated with 10 μg/ml goat anti-mouse IgM (Southern Biotechnology Associates) in PBS, 150 mM NaCl, and 0.1% Na_2CO_3. Plates were then blocked with PBS, 1% BSA, and 0.05% NaN_3. Three-fold serial dilutions of mouse sera and cell supernatant in PBS, 1% BSA, and 0.05% NaN_3 were added to the wells. Standard Abs for ELISA quantification were purified 3-83IgM from a transfectoma cell line, 3-83IgG2a from the HB-20 cell line (American Type Culture Collection) and mouse IgMA (Southern Biotechnology Associates) starting at a 1 μg/ml concentration. The plates were washed three times with PBS/0.5% Tween 20 and then detected depending on the ELISA. The 3-83IgM Abs were detected with biotin-conjugated 54.1 (30), followed by streptavidin-alkaline phosphatase (AP); Southern Biotechnology Associates. Total IgM and IgG Abs were detected with AP-conjugated goat anti-mouse IgM and goat-anti-mouse IgG (Southern Biotechnology Associates), respectively. The reactivity of mouse sera against H-2K^b was analyzed on plates coated with soluble H-2K^d (33). Bound anti-H-2K^b Abs were detected with AP-conjugated anti-IgM Abs. ELISA plates were developed by the addition of AP’s substrate (Sigma-Aldrich) solubilized in 0.1 M diethanolamine and 0.02% NaN_3. Absorbance values at 405 nm wavelength were obtained by reading the plates on a Wallac Victor (2) (PerkinElmer), a Titer-Tek Multiskan Plus (Labsystems), or a Versamsa (Molecular Devices) ELISA reader. Data were plotted in Microsoft Excel.

B cell hybridomas

For the generation of LPS-induced hybridomas, spleen cells were cultivated in vitro for 3 days in complete RPMI 1640 medium with 10% FCS and 20 μg/ml LPS (Sigma-Aldrich) before cell fusion. For the generation of stable hybridomas, cells were fused with SP2/0 myeloma cells in a 3–5:1 ratio, centrifuged, and resuspended in 50 μl of complete RPMI 1640 medium without FBS. Then, 0.5 ml of polyelectrolyte glycol 1450 (American Type Culture Collection) was added dropwise to the cell mix while shaking, and the cells were incubated for 2 min in the solution followed by centrifugation for 3 min at 37°C in 5% CO_2. Three-fold serial dilutions of mAbs in PBS, 1% BSA, and 0.05% NaN_3 were added to the wells. Supernatants from cultured cells were analyzed by ELISA after 7 days of culture. Cell cultures with PP2 and bafilomycin were set up as follows. The plates were plated in 10 96-well plates at limiting dilutions in complete RPMI 1640 medium with 15% FBS. To achieve hybridoma cloning, each plate was seeded with cells diluted 2-fold from those seeded in the previous plate until 10 plates were generated. Plates were incubated at 37°C in 5% CO_2 for 24–48 h before starting selection with 14.1 μg/ml hypoxanthine (Sigma-Aldrich) and 0.5 μg/ml azaserine (Sigma-Aldrich). Supernatants from wells with growing hybridomas (selected from 96-well plates in which <70% of wells had growing cells) were analyzed by ELISA for 3-83IgM and total IgM.

Results

Levels of 3-83 serum autoantibodies increase with age

We and others have previously shown that in 3-83IgI,H-2^d mouse, in which 3-83IgI^+ B cells develop in the presence of the H-2K^b autoantigen, virtually no B cells (<1%) retain surface 3-83Ig expression based on anti-idiotypic staining (23–25). Accordingly, 3-83IgI,H-2^d mice have not been found to harbor detectable 3-83 Ab in their sera (23–25). However, during a longitudinal study of

3 Abbreviation used in this paper: AP, alkaline phosphatase.
a 3-83Igi autoreactive mouse, we found that 3-83IgM autoantibodies were detectable in every 3-83Igi,H-2b mouse tested by 7 mo of age. These autoantibody levels also steadily increased in concentration with age, reaching levels of 10–140 μg/ml in 3-83Igi,H-2b, but not wild-type, mice older than 1 year (Fig. 1A and data not shown). Serum levels of 3-83Ig were analyzed with the anti-idiotypic 54.1 Ab (30), which is highly specific for the combination of 3-83H and 3-83κ chains (Fig. 3, D and E). The reactivity of sera from old 3-83Igi,H-2b mice toward the original H-2Kb autoantigen was also confirmed with an ELISA using soluble H-2Kb molecules (Fig. 1B).

Mechanisms of central tolerance can fail in developing B cells reacting with high avidity autoantigens

The presence of 3-83IgM in sera of old 3-83Igi,H-2b autoreactive mice suggested that at least some 3-83Ig+ B cells escaped central and peripheral tolerance and differentiated into IgM-secreting plasma cells despite expressing a high avidity autoreactive Ag receptor. However, consistent with what was previously reported for young adult mice, we were unable to detect B cells expressing 3-83Ig receptors in old 3-83Igi,H-2b mice (data not shown). The inability to detect 3-83Ig+ cells did not result from epitope masking, because the 54.1 anti-idiotypic (anti-3-83IgH + 3-83κ) Ab (30) was able to recognize surface-bound 3-83Ig even when the Ab was engaged with its cognate H-2Kb Ag (Fig. 2). Attempts to use the 54.1 anti-idiotypic Ab for intracellular detection of 3-83Ig resulted in nonspecific staining, which was also present in wild-type negative control cells (data not shown).

We reasoned that if 3-83Ig-bearing B cells were present, H-2Kb binding to 3-83Ig would probably induce the rapid internalization of 3-83Ig due to the high avidity interaction between these molecules. To examine this possibility we established a system to look for surface 3-83Ig expression on B cells once the presence of autoantigen was removed. Thus, adoptive transfers were established in which bone marrow cells of 4- to 6-wk-old 3-83Igi,H-2b (nonautoreactive) mice were transferred into lethally irradiated H-2b recipient mice, and the donor B cells that developed in this autoreactive context were subsequently analyzed for 3-83Ig surface expression upon removal from the host autoreactive background. In these chimeric mice, donor 3-83Ig+ H-2b B cells develop in the presence of H-2Kb molecules expressed by recipient stromal cells (rH-2Kb), and undergo B cell tolerance to this Ag similar to the intact 3-83Igi,H-2d mice, although the B cells do not express the autoantigen themselves (24, 25). Thus, spleen B cells from bone marrow chimera mice that have completed the process of central and peripheral tolerance can be isolated and examined for 3-83Ig surface expression in vitro in the absence of the H-2Kb autoantigen. For these experiments, spleen B cells from chimeric mice were typically cultured for 2 days in the absence of autoantigen and subsequently assessed for 3-83Ig surface expression.

We found that <4% (3.83 ± 1.16%) of the splenic IgM+ B cells that developed from the transfer of 3-83Igi,H-2d bone marrow into rH-2Kb hosts expressed surface 3-83Ig ex vivo (Fig. 3A, ex vivo, and Fig. 4). In contrast, after 2 days of culture in the absence of the H-2Kb autoantigen, we detected surface 3-83Ig expression in 21.9 ± 5.2% of B cells (Fig. 3A, in vitro, and Fig. 4). This frequency of 3-83Ig+ B cells was significantly >5.1 ± 2.9% (n = 7) found under similar conditions in a population of wild-type 3-83Ig- B cells (Fig. 3A, in vitro, and Fig. 4), representing background Ab staining. To ensure that the presence of 3-83Ig+ B cells was not an artifact of the in vitro culture system, we also determined the frequency of bone marrow chimera B cells that re-expressed 3-83Ig in vivo after adoptive transfer into wild-type and nonautoreactive H-2IgM+ recipient mice. In this study, 3-83Ig expression was analyzed on donor IgM+ B cells, thus excluding from the analysis recipient IgM+ B cells. When adoptively transferred into recipient H-2IgM+ mice, we found that 29.3 ± 3.3% (n = 5) of 3-83Ig+ bone marrow chimera IgM+ B cells expressed 3-83Ig on the surface 1 day after transfer, and the expression was 3-fold greater compared with wild-type mice (9.3 ± 3.6%; n = 5; Fig. 3A, in vivo). Thus, these results indicate that ~15–20% of 3-83Igi,H-2d,rH-2Kb spleen B cells maintain the potential to express the 3-83 Ab after undergoing negative selection during development, but do not express the autoantibody on the cell surface at detectable levels.

FIGURE 1. Autoantibodies accumulate in old autoreactive mice. A, Sera were collected from a group of wild-type (□, n = 9) and 3-83Igi,H-2d (□, n = 11) mice at 3, 7, and 12 mo of age. Serum from 3-83Igi,H-2d (□; n = 10) mice was collected only at ~3 mo of age. The 3-83IgM serum concentration was assessed by ELISA compared with a purified 3-83IgM standard of known concentration. Note that 3-83IgM levels in sera of 3-mo-old 3-83Igi,H-2b and wild-type mice were below detection (△). B, Serum IgM Ab was analyzed for reactivity against H-2Kb by ELISA on plates coated with soluble H-2Kb. Serum samples were from one wild-type (wt), two autoreactive 3-83Igi,H-2b, and one nonautoreactive 3-83Igi,H-2d mice. Plotted are values of OD405 vs serum dilution factors.

FIGURE 2. The 3-83 Ab interaction with the H-2Kb autoantigen does not abolish the 54.1 recognition of the 3-83Ig epitope. Spleone from 3-83Igi,H-2b nonautoreactive mice were preincubated, or not, with soluble H-2Kb-IgG1. Cells were subsequently stained with anti-IgG2b, 3-83Ig, and IgA Abs. Anti-IgG Abs reveal both IgM expression (<2% of B cells) and bound H-2Kb-IgG1A molecules. Intact and dotted lines in left and right panels represent B220+ gated B cells preincubated, or not, with Kb-IgG1 molecules, respectively. Left panel, Analysis of anti-IgM staining (IgM arrow indicates B cells expressing IgM; K′ arrow indicates B cells binding H-2Kb-IgG1A molecules). Right panel, Analysis of anti-3-83Ig staining.
Homozygosity of the 3-83 IgH and 3-83 Igk alleles in the 3-83Igi strain is required for the establishment of allelic exclusion in the absence of autoantigen (24, 26). It has been previously proposed that this phenotype results from either low expression of the targeted alleles or low affinity of 3-83 Abs for H-2Kd (24, 26), but the bases for this finding have not been definitively established. Nevertheless, to exclude that the persistence of 3-83Ig/H11001 B cells in 3-83Igi,H-2d,rH-2Kb bone marrow chimera mice was caused by the homozygous configuration of the Ig alleles, maintenance and re-expression of the autoreactive receptor were also assessed in mice that retained wild-type nonrearranged IgH and Igk alleles, i.e., in IgH3-83/Igh3-83/Igh3-83 (3-83Igi+/H11001) hemizygous animals. Using a similar strategy to that described above, we found that after removing the cells from the presence of autoantigen, 16.4 ± 4.3% (n = 3) of spleen IgM+ B cells from hemizygous 3-83Igi/H11001, H-2d,rH-2Kb bone marrow chimera mice were capable of expressing surface 3-83Ig (Figs. 3B and 4), a frequency well above that found in wild-type negative control animals (2.9 ± 0.8%; n = 3) in similar experiments and only slightly reduced compared with that in homozygous 3-83Igi mice (Fig. 4). Moreover, when cultured in the presence of LPS for 7 days, B cells from both homozygous 3-83Igi,H-2d,rH-2Kb (Fig. 3C) and hemizygous 3-83Igi+/H-2d,rH-2Kb (data not shown) bone marrow chimera mice secreted low, but detectable, amounts of 3-83IgM and total IgM by ELISA. The graph represents the arithmetic mean and SD of Abs in micrograms per milliliter. Below detection.

**FIGURE 3.** Receptor editing does not eliminate the genes encoding the autoantibody in a fraction of developing autoreactive B cells. A, Frequency of 3-83IgM+ B cells in 3-83Igi mice. Spleen cells from bone marrow chimeras or intact mice (as indicated) were analyzed ex vivo (top panels) for B220, IgM, and 3-83H+3-83k expression. Sorted B220+ (H-2d) cells from the same mice were similarly analyzed after 2 days of culture with LPS (in vitro; middle panels). Cells in dot plots of top and middle panels were gated for B220 expression. Sorted B220+ (IgM+) cells from the mice were also injected i.v. into CB17 H-2d,IgMb mice and analyzed after 24 h (in vivo; bottom panels) for B220, IgM+, and 3-83H+3-83k expression. Expression of 3-83H+3-83k in IgM+ B220+ donor cells is shown in the bottom panels. B220+ IgM+ host cells were excluded from this analysis. Numbers represent the proportion of cells in quadrants or histograms. B, Frequency of 3-83IgM+ B cells in 3-83Igi+/H11001 hemizygous mice. B220+ spleen cells from the indicated bone marrow chimeras and intact mice were cultivated in vitro in the presence of LPS. After 2 days of culture, cells were analyzed by flow cytometry for the expression of B220, IgM, and 3-83H+3-83k. B220+ gated cells are shown. Numbers represent the proportion of cells in quadrants. C, Analysis of secreted 3-83IgM. B220+ purified cells from wild-type (wt; n = 3) and 3-83Igi,H-2d (H-2d; n = 3) intact mice and 3-83Igi,H-2d,rH-2k bone marrow chimera (H-2b; n = 3) were cultured for 7 days in the presence of LPS. Cell culture supernatants from day 7 were analyzed for the presence of 3-83IgM and total IgM by ELISA. The graph represents the arithmetic mean and SD of Abs in micrograms per milliliter. Below detection. D, Flow cytometric analysis of spleen cells from a 3-83Hi (Igh3-83/Igh3-83, Igk+/+), Igk+/+ mouse stained for IgM and 3-83H+3-83k. Cells in lymphoid and living (propidium iodide-negative) gates are shown. Numbers represent the proportion of cells in quadrants. E, Analysis of 3-83IgM in sera of 3-83Hi mice. The levels of 3-83IgM in sera from the indicated mice were analyzed by ELISA. Plotted are OD405 vs serum dilution factors.

Homozygosity of the 3-83Igh and 3-83Igk alleles in the 3-83Igi strain is required for the establishment of allelic exclusion in the absence of autoantigen (24, 26). It has been previously proposed that this phenotype results from either low expression of the targeted alleles or low affinity of 3-83 Abs for H-2Kd (24, 26), but the bases for this finding have not been definitively established. Nevertheless, to exclude that the persistence of 3-83Ig+ B cells in 3-83Igi,H-2d,rH-2Kb bone marrow chimera mice was caused by the homozygous configuration of the Ig alleles, maintenance and re-expression of the autoreactive receptor were also assessed in mice that retained wild-type nonrearranged Igh and Igk alleles, i.e., in Igh3-83/Igh3-83, Igk3-83/Igk3-83 (3-83Igi+/H11001) hemizygous animals. Using a similar strategy to that described above, we found that after removing the cells from the presence of autoantigen, 16.4 ± 4.3% (n = 3) of spleen IgM+ B cells from hemizygous 3-83Igi+/H-2d,rH-2Kb bone marrow chimera mice were capable of expressing surface 3-83Ig (Figs. 3B and 4), a frequency well above that found in wild-type negative control animals (2.9 ± 0.8%; n = 3) in similar experiments and only slightly reduced compared with that in homozygous 3-83Igi mice (Fig. 4). Moreover, when cultured in the presence of LPS for 7 days, B cells from both homozygous 3-83Igi,H-2d,rH-2Kb (Fig. 3C) and hemizygous 3-83Igi+/H-2d,rH-2Kb (data not shown) bone marrow chimera mice secreted low, but detectable, amounts of 3-83IgM and total IgM by ELISA. The graph represents the arithmetic mean and SD of Abs in micrograms per milliliter. Below detection. D, Flow cytometric analysis of spleen cells from a 3-83Hi (Igh3-83/Igh3-83, Igk+/+), Igk+/+ mouse stained for IgM and 3-83H+3-83k. Cells in lymphoid and living (propidium iodide-negative) gates are shown. Numbers represent the proportion of cells in quadrants. E, Analysis of 3-83IgM in sera of 3-83Hi mice. The levels of 3-83IgM in sera from the indicated mice were analyzed by ELISA. Plotted are OD405 vs serum dilution factors.
3-83IgM. The detection of 3-83Ig was specific for the 3-83 H and 3-83κ L chain combination, because B cells and sera from 3-83Hi (Igh3-83+/−, Igk+/−) mice in which the 3-83H chain pairs with endogenous (and not 3-83κ) L chains had undetectable levels of 3-83Ig, similar to wild-type mice (Fig. 3, D and E). Although sera of young 3-83Ig+,H-2b mice did not have detectable levels of 3-83IgM Ab (data not shown), the presence of these autoantibodies in old 3-83Ig+,H-2b mice was not tested.

To confirm that 3-83Ig+ B cells also exist in intact 3-83Ig+,H-2b mice and are not specific to the bone marrow chimera system, B cell hybridomas were generated from LPS-induced spleen cells from 2- to 4-mo-old 3-83Ig+,H-2b homozygous and 3-83Ig+,H-2b hemizygous intact mice. The hybridomas were screened for the presence of secreted 3-83IgM and total IgM in the culture supernatants. We found that 60.2% of these autoantibodies in old 3-83Ig+,H-2b mice (data not shown). Moreover, similar frequencies of 3-83Ig+ B cell hybridomas were obtained from young and old 3-83Ig+,H-2b mice (Table I). Similar frequencies of 3-83Ig+ B cell hybridomas were obtained from young and old 3-83Ig+,H-2b mice (data not shown). Moreover, similar frequencies of 3-83Ig+ B cell hybridomas were derived from CD23hiCD21lo fo llicular and CD23loCD21hi marginal zone/transitional B cell subsets of 3-83Ig+,H-2b homozygous mice (Table I). These findings confirm that a fraction of the mature B cells in intact 3-83Ig+,H-2b mice retain the 3-83 Ab and that 3-83Ig+ autoreactive B cells are not confined to the marginal zone, in contrast with other models (17).

The discrepancy in the frequency of 3-83Ig+ B cells measured in hybridomas and in vitro analyses may be indicative of an increased propensity of 3-83Ig+ B cells to form hybridomas. Indeed, we found that spontaneous 3-83IgM− B cell hybridomas could easily be generated from nonstimulated B cells of 3-83Ig+,H-2b mice (Table I).

In summary, these data indicate that a significant fraction (10–20%) of 3-83Ig+ B cells fail to undergo central tolerance when developing in the presence of the high avidity H-2Kb autoantigen in both young and old 3-83Ig+ mice and differentiate into mature autoreactive B cells that do not express the autoreactive receptor on the cell surface.

Development of autoreactive 3-83Ig+ B cells depends on receptor editing and expression of nonautoactive Abs

The development of autoreactive B cells occurs through the expression of additional nonautoactive Abs by the same cells in some mouse models (17, 18, 34, 35). To identify whether this mechanism operates during development of 3-83Ig+ autoreactive B cells, the 3-83Ig+ mice were bred onto a Rag1-deficient background. It should be noted that receptor editing is completely impaired in the absence of Rag1, and 3-83Ig+,Rag1−/− B cells can only express 3-83 Abs. Flow cytometric analyses indicated that B220+CD22low immature B cells were observed in the bone marrow of 3-83Ig+,H-2b,Rag1−/− mice. However, transitional 1 CD23−CD21−, transitional 2, and mature CD23+CD21+ B cells were absent in the spleens of Rag1-deficient, but found in Rag1-sufficient, 3-83Ig+,H-2b mice (Fig. 5). These data indicated that the development of transitional and mature (folicular and marginal zone) autoreactive 3-83Ig+ B cells in 3-83Ig+,H-2b mice absolutely depends on receptor editing and the rearrangement of an endogenous Ig gene. This finding also suggested that receptor editing in 3-83Ig+,H-2b mice leads to Ig allelic/isotypic inclusion and the development of B cells that simultaneously express 3-83H, 3-83κ, and an

![FIGURE 4. Frequency of 3-83Ig+ B cells in various mouse strains. This is a summary of the experiments described in Figs. 3a and 6a in which sorted B220+ B cells from 3-83Ig+,H-2b (n = 5), 3-83Ig+,H-2b (n = 5), and B1-83-Ig+IgM−,H-2b (n = 4) bone marrow chimeras and from wild-type (n = 7) and 3-83Ig+,H-2b (n = 3) intact mice were cultivated for 2 days in the presence of LPS and then analyzed for the expression of B220, IgM, and 3-83IgM+3-83κ. The graph indicates the arithmetic mean and SD of the frequencies of 3-83IgM+ cells in the total IgM+ B cell populations.](http://www.jimmunol.org/)
additional Ig H or L chain. In support of this conclusion, we found that a significant fraction of 3-83Ig⁺ hybridomas coexpressed Igλ (Table I). Notably, eight of 24 (33.3%) and 14 of 24 (58.3%) LPS-induced hybridomas from 3-83Ig⁺,H-2b homozygous and 3-83Ig⁺,H-2b hemizygous mice, respectively, expressed both 3-83IgM (3-83H and 3-83κ) and Igλ (Table I).

To determine whether concomitant expression of a nonautoreactive Ag receptor in developing 3-83Ig⁺ autoreactive B cells is sufficient to mediate their development, we bred 3-83Ig to B1-8H3-83x (Igh⁺[Iγk]),[3-83] (23) mice. Through this breeding, we generated a mouse strain, B1-8/3-83Igi (Igh⁺[Iγk],3-83Ig⁺,H-2d) in which all immature B cells express the 3-83 and B1-8 Ig H chains in addition to the 3-83Ig κ-chain, which pairs with both H chains at similar levels (Fig. 6C and data not shown). We have previously established that the B1-8H3-83κ Ab is nonautoreactive and mediates the development of B cells in various genetic backgrounds (23). In fact, every B cell of B1-8/3-83Igi mice coexpresses B1-8H3-83κ and 3-83H3-83κ BCRs in the absence of the H-2K⁺ Ag (chimeric B1-8H/3-83H3-83κ receptors may also be expressed; Fig. 6C and data not shown).

To determine the frequency of B cells that retain expression of the 3-83Ig autoantibody in B1-8/3-83Igi,H-2b mice, we generated chimeric H-2b recipient mice through adoptive transfer of bone marrow cells from 4- to 6-wk-old B1-8/3-83Igi,H-2d donor animals (B1-8/3-83Igi,H-2d,rH-2K⁺ chimeras). B cells from B1-8/3-83Igi,H-2d,rH-2K⁺ chimeras were isolated and analyzed for 3-83Ig surface expression after 2 days of culture. We found that 45.0 ± 5.0% (n = 4) of cultured IgM⁺ B cells from B1-8/3-83Igi,H-2d,rH-2K⁺ chimeras retained the 3-83 Ab (Figs. 4 and 6A, in vitro), and 3-83Ig was clearly and reproducibly expressed to a significant level and above the background detected in wild-type mice (5.1 ± 2.9%; n = 7). Importantly, 3-83Ig surface re-expression was never observed in cultured B cells from intact B1-8/3-83Igi,H-2b mice, which also express the autoantigen (data not shown). Compared with the frequency of 3-83Ig⁺ B cells in 3-83Igi,H-2b mice, these data indicate that the probability that 3-83Ig⁺ autoreactive immature B cells further differentiate into splenic B cells is ~2-fold higher when the cells are provided during their development with a preformed nonautoreactive Ab (Fig. 4). These findings, therefore, indicate that the expression of a nonautoreactive Ag receptor is sufficient to promote the development of cells that also express a high avidity autoreactive Ab.

The fact that not all spleen B cells from B1-8/3-83Igi,H-2d,rH-2K⁺ mice retained apparent expression of 3-83Ig suggested that receptor editing may have also been induced in these mice, resulting in deletion of the 3-83Ig gene(s) in some cells. Flow cytometric analysis of Igλ expression was performed to determine whether B1-8/3-83Igi⁻ B cells undergo receptor editing at the Ig L chain loci during their development in the presence of the H-2K⁺ autoantigen. This analysis demonstrated a high frequency (30–40%) of λ-expressing cells in the B cell population of B1-8/3-83Igi,H-2b mice (Fig. 6B), which is a hallmark of receptor editing in this system (23, 36). These data indicated that 3-83Ig⁺ developing B cells undergo receptor editing despite the expression of the nonautoreactive B1-8H,3-83κ Ag receptor. Flow cytometric analysis of IgM and Id expression in bone marrow cells demonstrated that immature B1-8/3-83Igi B cells down-regulate the autoreactive 3-83H,3-83κ Ag receptors in the presence of the H-2K⁺ autoantigen while maintaining the nonautoreactive B1-8H,3-83κ Ag receptors on the surface (Fig. 6C). Importantly, these data indicate that surface expression of Ag receptors that do not engage self-Ags does not prevent the induction of receptor editing in immature B cells that have bound autoantigen.

**FIGURE 6.** Expression of a nonautoreactive BCR is sufficient to promote the development of autoreactive 3-83Ig⁺ B cells. A, Bone marrow chimeras were generated with the indicated donor and recipient mice. Spleen cells were analyzed ex vivo (top panels) for B220, IgM, and 3-83H⁺+3-83κ expression. Sorted B220⁺ (H-2⁺) cells from bone marrow chimeras were similarly analyzed after 2 days of culture with LPS (in vitro; lower panels). B220⁺ gated cells are shown. Numbers represent the proportion of cells in quadrants. B and C, Spleen (B) and bone marrow (C) cells from the indicated intact mice were analyzed for B220, Igλ (B), B220, IgM, B1-8H⁺+L (C, upper panels), and B220, IgM, 3-83H⁺+3-83κ (C, lower panels) expression. Cells shown in C were gated for B220 expression. Numbers indicate the relative frequencies of cells in quadrants.

**Autoreactive BCR is internalized after Src family kinase-dependent phosphorylation and is probably degraded inside the cell**

As shown above, B cells that retain expression of the 3-83 autoreactive Ab do not express it on the cell surface unless removed from the presence of the autoantigen. Internalization of Ag-engaged BCR has been shown to require phosphorylation of the BCR Ig-α molecule (37), which is mediated by Src and Syk family kinases, and inhibition of Src family kinases has been shown to diminish internalization of engaged BCR in cell lines (38, 39). To
test whether autoreactive 3-83 Ag receptors were internalized as a result of autoantigen engagement and consequent to receptor phosphorylation, surface 3-83Ig expression was analyzed in B1-8/3-83Ig,H-2b spleen B cells treated with the Src family kinase inhibitor PP2. As shown (Fig. 7), levels of surface 3-83Ig in B1-8/3-83Ig,H-2b cells and, consequently, the frequency of cells expressing detectable surface 3-83Ig amounts significantly increased upon PP2 treatment despite the continued presence of the autoantigen. Thus, PP2 treatment inhibits 3-83 BCR internalization on B1-8/3-83Ig,H-2b spleen B cells and partially restores 3-83Ig surface expression. These findings demonstrate that the 3-83 autoantibody is synthesized in the presence of the H-2Kb autoantigen and indicate that 3-83 BCR is internalized after Src-mediated tyrosine phosphorylation of the Ag receptor and/or other relevant molecules.

By using flow cytometric, immunohistological, and biochemical approaches, we were unable to detect 3-83 Abs within 3-83Ig,H-2b and B1-8/3-83Ig,H-2b B cells (data not shown). Therefore, we speculated that internalized autoreactive Abs are quickly degraded in the endosome/lysosome compartment and never accumulate to detectable amounts. To test this assumption, levels of surface 3-83Ig expression were assessed in B1-8/3-83Ig,H-2b spleen B cells treated with bafilomycin, a lysosome inhibitor, in combination with PP2. The levels of 3-83Ig surface expression were higher in cells treated with both PP2 and bafilomycin than in cells treated with PP2 alone (Fig. 7). Treatment of cells with bafilomycin alone did not alter 3-83Ig expression compared with that in cells treated with DMSO (data not shown). Notably, treatment with PP2 and bafilomycin allowed the detection of a population of surface 3-83Ig- B cells at a frequency close to that determined with the bone marrow chimera studies. These data suggest that autoreactive 3-83Ig Abs are degraded within the endosome/lysosome compartment after endocytosis, and that inhibition of the lysosome-dependent degradation pathway results in recycling of the internalized Ab onto the cell surface.

**Discussion**

Autoreactive B cells specific for bone marrow Ags bypass mechanisms of central tolerance when expressing low avidity BCRs or when carrying mutations that impair BCR signaling or inhibit cell death (20, 40, 41). A large body of data, however, has indicated that high avidity autoreactive B cells (e.g., those reacting with membrane-bound Ags) succumb to central tolerance, which prevents their differentiation and migration into the peripheral compartment (20, 25, 42).

We have recently shown that every immature B cell reactive with membrane-bound autoantigens changes their Ag specificity through the receptor-editing process (25). Despite this finding, we demonstrate in this study that receptor editing is unable to delete the gene(s) encoding the autoantibody in a significant fraction of these cells. In fact, B cells reacting with a ubiquitous membrane-bound autoantigen were found in the spleens of mice retaining expression of the autoreactive Ab. Thus, our data demonstrate that a proportion of high avidity autoreactive B cells can differentiate and survive central tolerance, avoiding both the removal of the genes encoding the autoantibody mediated by receptor editing and apoptosis through clonal deletion. These high avidity autoreactive B cells do not expose the autoreactive BCR on the cell surface at detectable levels, although they do express normal levels of BCRs with innocuous specificity. We believe that for this reason the development of high avidity autoreactive B cells has not been previously observed. Thus, these autoreactive B cells look like nonautoreactive cells as they conceal their self-reactive Abs.

Our findings demonstrate that receptor editing is an absolute requirement for the development of autoreactive B cells specific for a ubiquitous membrane-bound autoantigen. This is well illustrated by the absence of transitional and mature B cells in spleens of 3-83Ig,H-2b,Rag1-deficient mice that cannot undergo receptor editing (Fig. 5) and was previously shown for conventional 3-83Ig transgenic mice on a Rag1-deficient background (43). The requirement of receptor editing is probably related to the fact that this process can break allelic/isotypic exclusion and provide a novel, nonautoreactive Ag receptor to developing autoreactive B cells. In fact, when developing autoreactive B cells were provided with prerearranged genes encoding a nonautoreactive BCR, they differentiated and migrated into the spleen in higher numbers. Therefore, receptor editing is the mechanism that ensures elimination of most autoreactive specificities, but it is also the process that allows a small fraction of autoreactive B cells to reach the spleen. Moreover, the expression of a nonautoreactive BCR is sufficient to promote the development of high avidity autoreactive B cells.

The development of dual Ab-expressing B cells that retain an autoreactive specificity has been previously observed in anti-DNA IgM chain knockin mice (17, 18) in which extensive receptor editing generates dual Ab mature B cells expressing autoreactive and nonautoreactive L chains. In these mice, however, both autoreactive and nonautoreactive BCRs are expressed on the cell surface (17), perhaps indicating that the autoreactive Ab on these cells displays low avidity for self-Ags. Our data, therefore, extend these
previous findings to demonstrate that the expression of nonautoreactive BCRs can drive the differentiation of autoreactive B cells that express high avidity BCRs.

Both Ag-engaged and nonengaged BCRs transduce signals in B cells. Ag binding and signaling by autoreactive BCRs on immature B cells have been shown to promote arrest of B cell differentiation (44, 45) and induction of Rag expression and receptor editing (46, 47). In contrast, the presence of a basal tonic signal delivered by presumably unbound (i.e., nonautoreactive) BCRs has been suggested to suppress Rag expression and mediate B cell differentiation and positive selection into the spleen (48, 49). We show that dual BCR-expressing (B1-8/3-83Igi,H-2b) immature B cells that simultaneously express autoreactive and nonautoreactive BCRs and, therefore, transmit both Ag-induced and tonic BCR signals undergo receptor editing as well as differentiation. Thus, receptor editing is induced despite the presence of nonautoreactive BCRs on the cell surface, and a significant fraction of cells also differentiate to mature B cells despite expressing autoreactive BCRs. This clearly indicates that signals transmitted via autoreactive BCRs (at least when diluted by nonautoreactive BCRs) are not necessarily dominant over tonic signals, whereas tonic signals can overcome those mediated by autoantigens. We found that despite differentiating in higher numbers, dual (B1-8/3-83Igi) Ab-expressing autoreactive B cells also undergo receptor editing. It is possible that the differentiation of these cells relies on receptor editing to provide additional Ig L chains that lead to increased expression of a nonautoreactive BCR (and of tonic signal) above a certain threshold. Indeed, in the presence of autoantigen, the levels of nonautoreactive receptors are, on the average, 60% of those in the absence of autoantigen (Fig. 6C: note the shift to the left of the IgM+ cell population in B1-8/3-83Igi,H-2b relative to B1-8/3-83Igi,H-2d bone marrow cells stained for B1-8H+) and, thus, may not be sufficient to suppress Rag expression. In addition, continued engagement of self-Ag results in the internalization of the autoreactive BCR (Fig. 6C), presumably terminating signals leading to Rag expression and ultimately allowing tonic signals to promote differentiation. Clearly, additional studies are required to understand the qualitative, quantitative, and functional differences between Ag-mediated and tonic BCR signals in B cell development and selection. In accord with recent published work (47, 49), our data suggest that these two signals have intrinsic qualitative differences that allow independent translation into distinct biological processes.

We show that treatment with Src kinase and lysosome/endosome inhibitors restores surface expression of the autoreactive BCR in the presence of the autoantigen. We interpret these findings to indicate that the autoreactive Ab is continually synthesized and assembled on the cell surface of peripheral B cells, but is rapidly internalized upon Ag binding and receptor phosphorylation by Src kinases. The increased expression of surface autoreactive BCR in cells treated with bafilomycin and PP2 compared with that in cells treated with PP2 alone suggests that the internalized receptor is degraded within the lysosome/endosome compartment. In the presence of bafilomycin, the internalized receptor is probably recycled back on the cell surface instead of being degraded, resulting in increased surface expression. Thus, continual autoantigen-mediated receptor internalization and degradation prevent cell surface 3-83 BCR from reaching detectable levels.

Src kinases are proximal signaling elements in the BCR signaling pathway that are activated by Ag binding and, consequently, phosphorylate the BCR as well as other pathway components (50). The fact that an Src kinase inhibitor restores surface 3-83Ig expression indicates that the autoreactive BCRs are constitutively signaling in the presence of autoantigen. This chronic BCR signaling may induce an anergic phenotype in dual Ab-expressing autoreactive B cells, which is consistent with the absence of serum 3-83Ig Abs in mice <7 mo of age. Because 3-83Ig+ autoreactive B cells express an additional innocuous BCR, our results also suggest that these nonautoreactive BCRs are nonfunctional in the context of an autoreactive cell, or that a mechanism exists that prevents differentiation of autoantibody-secreting plasma cells from dual reactive B cells, which has been suggested previously (17, 21, 51). In addition, serum isotype-switched 3-83 Abs were never detected (data not shown), even in autoreactive mice with significant levels of 3-83IgM, which suggests either an absence of T cell help or an inability of the autoreactive B cells to receive such help. At present, it is unclear why sera 3-83IgM autoantibodies become detectable only in mice >7 mo of age. We have found no difference in the frequency of 3-83Ig+ hybridomas obtained from spleens of old and young 3-83Igi,H-2b mice (data not shown), suggesting that 3-83Ig+ autoreactive B cells do not accumulate with age. It may be possible that 3-83Ig+ autoreactive B cells survive and accumulate in the B1 compartment in the peritoneal cavity. The frequency of B1-8 3-83Ig+ cells was similar in the B1 and B2 cell compartments of 3-83Igi,rH-2b bone marrow chimera mice (data not shown). Moreover, we found low, but similar, frequencies (<3%) of B1 cells in the lymphocyte population of the peritoneal cavity of young and old autoreactive and nonautoreactive 3-83Igi mice (data not shown), making it unlikely they represent the main source of 3-83 autoantibodies in old 3-83Igi,H-2b mice. Because 3-83Ig+ B cells were found in all B cell compartments (Table I and data not shown), it is presently unknown from which B cell type 3-83Ig+ plasma cells differentiate. A possibility under evaluation is that 3-83Ig+ long-lived plasma cells accumulate with age in 3-83Igi,H-2b mice. Nevertheless, it is evident that some mechanisms that regulate autoreactive B cells are suppressed with aging, and/or that progressive exposure to environmental Ags can break peripheral tolerance.

It is important to note that the generation of dual Ab-expressing B cells is not restricted to Ig knock-in mice. Although allelic and isotypic exclusion in wild-type mice may be more stringent than that in Ig transgenic and knock-in mice because of the epigenetic modifications that ensure a differential rate of accessibility at the Ig alleles during physiological B cell development (52), rare B cells carrying two productively rearranged L chain genes and cells expressing either two H or two L chains have been described in both wild-type mice and humans (6–13, 53, 54). The mechanism(s) that generates dual Ab B cells in wild-type and the nature of the Ag specificity of these cells have not been thoroughly investigated. Based on our observations and those of anti-DNA mice (17, 18), we propose that these cells probably express one autoreactive and one (or more) nonautoreactive specificity and have developed through the process of receptor editing. In support of this model, recent works from the Wabl and Nussenzweig groups (55–57) have identified the presence of dual Ab-expressing human and mouse B cells that carry an autoreactive specificity. Our work demonstrates that even high avidity specificities for membrane-bound self-Ags can be selected into the spleen by allelically/isotypically included B cells and that receptor editing is essential to this process. Importantly, we show that high avidity autoreactive B cells are superficially tolerant, because they conceal their autoantibodies within the cell, but can differentiate into autoantibody-secreting plasma cells through mechanisms that are still unclear, thus raising the possibility that dual Ab-expressing B cells may be the progenitors of autoantibody-forming cells in autoimmunity. It has long been speculated that polyreactive B cells that express Abs
that cross-react with foreign and self-Ags can be activated by microbacterial infections to differentiate into autoantibody-secreting cells. The finding of dual Ab-expressing B cells suggests that polyreactivity can also arise from the expression of multiple Ig H and L chains by the same B cell. Thus, dual Ab-expressing autoreactive B cells provide a mechanism by which autoantibody-forming cells may develop in response to infections. However, the development of autoimmunity probably depends on the additional contribution of defects in the tolerance program. Thus, it can be envisioned that defects during the central stages of B cell tolerance may favor the development of dual Ab-expressing autoreactive B cells, and that defects in the peripheral stages of B and, probably, T cell tolerance can lead to the activation of dual Ab-expressing autoreactive B cells and the secretion of self-reactive Abs that ultimately mediate autoimmunity.

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Disclosures

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